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Canberra**

I, LEANNE MYNOTT, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PQ 1645 for a patent by THE NATIONAL UNIVERSITY OF SINGAPORE filed on 15 July 1999.

I further certify that the above application is now proceeding in the name of INSTITUTE OF MOLECULAR AND CELL BIOLOGY pursuant to the provisions of Section 104 of the Patents Act 1990.

WITNESS my hand this
Eighth day of October 1999

LEANNE MYNOTT
TEAM LEADER EXAMINATION
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Institute of Molecular & Cell Biology

# AUSTRALIA

## Patents Act 1990

## PROVISIONAL SPECIFICATION

for the invention entitled:

"Therapeutic Compounds and Methods"

The invention is described in the following statement:

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# THERAPEUTIC COMPOUNDS AND METHODS

## FIELD OF INVENTION

5 The present invention relates generally to agents useful in the treatment or prophylaxis of viral mediated disease conditions. More particularly, the present invention provides therapeutic agents useful in the treatment of cervical cancer, genital warts or asymptomatic infections caused or otherwise exacerbated by a mammalian papillomavirus (MPV). The present invention is further directed to methods of treatment using said agents as well as
10 methods of identifying same.

## BACKGROUND OF THE INVENTION

Viral mediated disease conditions represent some of the most debilitating diseases affecting
15 humans and animals and are responsible for significant mortality and morbidity. This is particularly the case for cancers associated with viral transformation of host cells. One particularly serious form of cancer is cervical cancer. Persistent infection of the transformation zone of the cervix uteri with MPVs such as human papillomavirus (HPV) is seen as a primary cause of cervical cancer. Approximately half a million women die of
20 cervical cancer every year, while a much higher number of patients are exposed to preinvasive disease or genital warts, and one has to conclude that treatment of these virally caused neoplasias is still inadequate in spite of the long-term establishment of surgical techniques.

25 MPV genomes encode proteins with molecular properties required for cellular transformation in cell culture and *in situ* . Human papillomavirus-16 (HPV-16) is the most common HPV type in malignant neoplasia and is found in about 60% of all cervical carcinomas, while about twenty other HPV types account for another 30% of these malignancies. Other HPV types that infect genital mucosa or skin, like HPV-6 and HPV-11, are most often associated

with benign neoplasia, such as genital warts.

Current treatment for HPV-16 associated lesions is surgery, while limited success is achieved for HPV-6 and HPV-11 lesions with immune modulators like interferon. Prevention of infection by HPV by vaccination and challenge of established HPV infections by immune therapy are under intense investigation, but are presently not established clinical procedures.

A need exists, therefore, for further therapeutic agents useful in the treatment or prophylaxis of disease conditions caused or exacerbated by MPVs and for methods of identifying same.

## SUMMARY OF INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise" and variations such as "comprises" and "comprising" will be understood to imply the inclusion of a stated integer or step or group of integers but not the exclusion of any other integer or step or group of integers.

One aspect of the present invention provides an agent useful in the treatment or prophylaxis of a disease condition caused or exacerbated by an MPV, said agent comprising a compound capable of reducing, inhibiting or otherwise decreasing the activity of a protein encoded by an MPV gene where said agent facilitates disruption of a chelated metal cation domain present in said protein.

Still yet another aspect of the invention contemplates a composition comprising a compound capable of facilitating the disruption of a chelated metal cation domain of a protein encoded for by an MPV gene, together with a pharmaceutically acceptable carrier, diluent or excipient.

Yet a further aspect of the invention relates to a method of treating a disease condition caused or exacerbated by an MPV comprising the administration of an effective amount of a

compound capable of facilitating the disruption of a chelated metal cation domain of a protein encoded for by an MPV gene to a mammal in need thereof.

Preferably, the MPV is a human papilloma virus (HPV)

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## BRIEF DESCRIPTION OF THE FIGURES

Figure 1      diagrammatically depicts the E6 protein which consists of 158 amino acids, with two Cys-X2-Cys-X29-Cys-X2-Cys zinc fingers forming the most
10      conspicuous secondary structure. Amino acid residues shown by encircled letters are conserved among HPV-6, HPV-11, HPV-16 and HPV-18. HPV-16 and HPV-18 are the most prevalent papillomaviruses in carcinomas of the cervix precursor lesions.

15 Figure 2      graphically depicts the effective concentration for C16 under the experimental IVT-assay conditions for E6BP and E6AP. $^{35}$S-Cys E6 was incubated with the indicated concentrations of C16 and assayed for complex formation with E6BP or E6AP. GST reflects the background binding of IVT E6 protein on GST-beads.

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Figure 3      graphically depicts viability assays of HPV-infected cell lines incubated with C16 and azodicarbonamide (C4). All values were normalized to the values obtained in the presence of DMSO only.

25 Figure 4      photographically depicts viability of SiHa, HeLa, 444 and HaCat cells when treated with C16 compound.

Figure 5A      depicts p53 protein expression for the cell lines HeLa, SiHa, MCF7 and HaCat when treated with C16.

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Figure 5B      depicts the cleavage of poly-ADP ribose polymerase (PARP) in HeLa cells
incubated with C16 overnight but not C16 treated HaCat cells.

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The following single and three letter abbreviations are used for amino acid residues:

| Amino Acid | Three-letter Abbreviation | One-letter Symbol |
|---|---|---|
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Cysteine | Cys | C |
| Glutamine | Gln | Q |
| Glutamic acid | Glu | E |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |
| Any residue | Xaa | X |

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## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The agents of the present invention are especially useful in the treatment of disease conditions caused by an MPV such as HPVs, for example HPV-6, HPV-11, HPV-16, HPV-18.

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All HPVs have circular double stranded DNA genomes with sizes close to 8kb. The genomes of different HPV types can be aligned, and there are eight genes that are homologous among all genital HPV types. These genes contain many sequence similarities, which suggest similar and conserved (although not necessarily identical) functions. The transforming properties of

10 HPV-16 originate from three oncoproteins that are the products of the genes E5, E6, and E7. These proteins have pleotropic effects with consequences for transmembrane signalling, regulation of cell cycle, transformation of established cell lines, immortalization of primary cell lines, and chromosomal stability (1,2). The E6 oncoprotein can form a ternary complex with the cell cycle regulator p53 and E6 associated protein, E6AP, with the result of

15 degradation of p53 by the ubiquitination pathway (3,4). In another mechanism, the E6 protein can bind to E6BP (also called ERC-55), a calcium binding protein localized in the endoplasmic reticulum, with possible consequences for intracellular signalling (5). E6 changes cellular morphology, as it interacts with paxillin and thereby disrupts the actin cytoskeleton (6). E6 has also been described to activate (7,8) or, alternatively, repress

20 transcription (9), to stimulate telomerase (10), to immortalize primary cell cultures (1, 2) and to interfere with the differentiation of human keratinocytes (4).

The E6 protein of HPV-16 (Fig. 1) has a size of 158 amino acids. Its most conspicuous sequence motifs are two Cys-X2-Cys-X29-Cys-X2-Cys zinc fingers (11-13). Analysis of

25 Swiss-Prot database indicates that this sequence motif is unique for papillomavirus E6 and E7 proteins (14), and includes numerous specific amino acids residues, highly conserved among all carcinogenic HPVs as well as many animal and human papillomavirus associated with benign lesions. The homology between all papillomavirus E6 genes permits the alignment of their nucleotide sequences, forming a useful database to establish papillomavirus taxonomy

30 (15-17). A similar zinc finger is found in the E7 protein. The extreme conservation of E6

and E7 zinc fingers among viruses with otherwise significant sequence diversity suggests that this zinc-binding motif is required for the structure and the function of HPV E6 and E7 oncoproteins, and it has been shown that mutations affecting the HPV-16 and the bovine papillomavirus type 1 (BPV-1) E6 zinc fingers interfere with cellular transformation as well

5 as with complex formation between E6 and E6AP and E6BP.

The structure and function of the HPV-16 E6 oncoprotein depends on the integrity of the zinc fingers, in which the sulhydryl-groups of four cysteines serving as metal-chelating residues.

10 The precise role of E6 in the etiology of cervical cancer is difficult to assess directly, but rather has to be inferred mostly from information on E6 function in cell culture or animals systems or molecular studies in vitro. The presently available knowledge suggests functions of E6 (and E7) in situ in three different pathological scenarios. (i) In stratified epithelia, uninfected epithelial cells differentiate without further mitoses after they left the basal and

15 became part of the suprabasal layers. After infection by HPVs, E6 and E7 proteins interfere with this normal repression of mitosis. The consequence is a dedifferentiated and expanded cell population with HPV genomes and the progression from a clinically latent infection into a benign intraepithelial neoplasia. (ii) In these benign lesions, E6 and E7 maintain a high frequency of aberrant mitoses leading to chromosomal aberrations and aneuploidies, raising

20 the chance for generation of increasingly tumorigenic cellular variants (32). (iii) Continuous expression of E6 and E7 may be required for continuous proliferation of malignant tumours and metastases (33, 34, 24). Anti-E6 and anti -E7 drugs should desirably be able to interfere with HPV lesions on all three levels of carcinogenesis. Accordingly, the compounds described herein may be useful in therapeutic or prophylactic applications where these

25 pathological scenarios are implicated.

Accordingly, one aspect of the present invention provides an agent useful in the treatment or prophylaxis of a disease condition caused or exacerbated by an MPV, said agent comprising a compound capable of reducing, inhibiting or otherwise decreasing the activity of a protein

30 encoded by an MPV gene where said agent facilitates disruption of a chelated metal cation

domain present in said protein.

Preferably, the disease conditions to be treated are cervical cancer or precursor lesions of this malignant neoplasia, which are called cervical intraepithelial neoplasia (CIN) or squamous

5 intraepithelial lesions (SIL). The agent may also be useful in the treatment of asymptomatic infections of the cervix in patients identified by DNA diagnosis, or asymptomatic infections that are assumed to remain after surgical treatment of cervical cancer, CIN or SIL, or asymptomatic infections presumed to exist following epidemiological reasoning. (All of these conditions mentioned before are caused by HPV-16, HPV-18 and related viruses). The

10 disease conditions to be treated also include genital warts (caused most often by HPV-6 and HPV-11), and common warts and plantar warts (caused by HPV-2 and HPV-1). All of these conditions are also caused by a large number (presently 50) of other HPV types, and the agents may also be usefully directed against these viruses. All of these lesions presumable derive from asymptomatic infections, that are most often not diagnosed. It can be assumed

15 that the agents can also be usefully targetted against all of these asymptomatic infections.

As used herein, the term "chelated metal cation domain" refers to the structure of a protein molecule formed by chelation or association of a metal cation with two or more non-adjacent amino-acid residues. The amino acid residues may reside on a single protein molecule to form

20 a "finger" or, alternatively, reside on different protein molecules to form, for example, a dimer. In a preferred embodiment, the metal cation is selected from manganese, iron, cobalt, nickel, copper or zinc. Most preferably, the metal is zinc. In another embodiment, the metal cation is chelated to four amino acid residues. In yet another embodiment of the invention, the metal atom is chelated to at least one cysteine residue, preferably via the sulfhydryl group.

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In yet a more preferred embodiment the chelated metal cation domain is a zinc domain in which the sulfhydryl groups of four cysteine residues are chelated to the zinc cation. In still yet a more preferred embodiment, the zinc domain comprises the Cys-X2-Cys-X29-Cys-X2-

30 Cys sequence motif, wherein the zinc atom is chelated to the four Cys residues via the

sulfhydryl groups (see Figure 1).

As used herein, a protein molecule encoded for by an MPV gene refers to a peptide, polypeptide or other amino acid sequence translated from a gene in an MPV genome, or
5  derivative thereof. Preferably, the MPV is an HPV, more preferably HPV-16 or HPV-18. In a preferred embodiment the gene is HPV-16 E6, HPV-16 E7, HPV-18 E6 or HPV-18 E7. Most preferably the gene is HPV-16 E6. Preferably, the protein is the E6 or E7 oncoprotein.

Compounds which may be useful in the treatment of diseases and conditions caused by MPVs
10  include compounds of the general Formula (I)



(I)
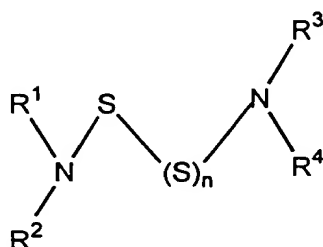
20  wherein

n is selected from 1-5

$R^1$ - $R^4$ are independently selected from hydrogen, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally
25  substituted arylalkyl optionally substituted acyl, optionally substituted heterocyclyl, halo alkyl, arylalkyl, carboxy, carboxy ester and carboxamido; or

$R^1$ and $R^2$ together, and/or $R^3$ and $R^4$ together, independently form a group of formula (a):

$$- (CH_2)_l - U_m - (CH_2)_n -　　　　(a)$$

wherein:　　U is selected from $CH_2$, O, NH or S;

l and n are independently selected from 0 to 6 and m is 0 or 1 when U is $CH_2$

and m is 1 when U is O, NH or S, such that

l+m+n is greater than or equal to 2;

and wherein any one or more ($CH_2$) or NH groups may be further optionally substituted.

As used herein the term "alkyl", denotes straight chain, branched or cyclic fully saturated hydrocarbon residues. Unless the number of carbon atoms is specified the term preferably refers to $C_{1-20}$ alkyl or cycloalkyl. Examples of straight chain and branched alkyl include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, amyl, isoamyl, sec-amyl, 1,2-dimethylpropyl, 1,1-dimethyl-propyl, hexyl, 4-methylpentyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 1,1-dimethylbutyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2,-trimethylpropyl, 1,1,2-trimethylpropyl, heptyl, 5-methoxyhexyl, 1-methylhexyl, 2,2-dimethylpentyl, 3,3-dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-dimethyl-pentyl, 1,2,3,-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-trimethylbutyl, octyl, 6-methylheptyl, 1-methylheptyl, 1,1,3,3-tetramethylbutyl, nonyl, 1-, 2-, 3-, 4-, 5-, 6- or 7-methyl-octyl, 1-, 2-, 3-, 4- or 5-ethylheptyl, 1-, 2- or 3-propylhexyl, decyl, 1-, 2-, 3-, 4-, 5-, 6-, 7- and 8-methylnonyl, 1-, 2-, 3-, 4-, 5- or 6-ethyloctyl, 1-, 2-, 3- or 4-propylheptyl, undecyl, 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8- or 9-methyldecyl, 1-, 2-, 3-, 4-, 5-, 6- or 7-ethylnonyl, 1-, 2-, 3-, 4- or 5-propylocytl, 1-, 2- or 3-butylheptyl, 1-pentylhexyl, dodecyl, 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9- or 10-methylundecyl, 1-, 2-, 3-, 4-, 5-, 6-, 7- or 8-ethyldecyl, 1-, 2-, 3-, 4-, 5- or 6-propylnonyl, 1-, 2-, 3- or 4-butyloctyl, 1-2-pentylheptyl and the like. Examples of cyclic alkyl include mono- or polycyclic alkyl groups such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclononyl, cyclodecyl and the like.

As used herein the term "alkenyl" denotes groups formed from straight chain, branched or

cyclic hydrocarbon residues containing at least one carbon-carbon double bond including ethylenically mono-, di- or poly-unsaturated alkyl or cycloalkyl groups as previously defined. Unless the number of carbon atoms is specified the term preferably refers to $C_{1-20}$ alkenyl. Examples of alkenyl include vinyl, allyl, 1-methylvinyl, butenyl, iso-butenyl, 3-methyl-2-

5 butenyl, 1-pentenyl, cyclopentenyl, 1-methyl-cyclopentenyl, 1-hexenyl, 3-hexenyl, cyclohexenyl, 1-heptenyl, 3-heptenyl, 1-octenyl, cyclooctenyl, 1-nonenyl, 2-nonenyl, 3-nonenyl, 1-decenyl, 3-decenyl, 1,3-butadienyl, 1-4,pentadienyl, 1,3-cyclopentadienyl, 1,3-hexadienyl, 1,4-hexadienyl, 1,3-cyclohexadienyl, 1,4-cyclohexadienyl, 1,3-cycloheptadienyl, 1,3,5-cycloheptatrienyl and 1,3,5,7-cyclooctatetraenyl.

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As used herein the term "alkynyl" denotes groups formed from straight chain, branched or cyclic hydrocarbon residues containing at least one carbon-carbon triple bond including ethynically mono-, di- or poly- unsaturated alkyl or cycloalkyl groups as previously defined. Unless the number of carbon atoms is specified the term preferably refers to $C_{1-20}$ alkynyl.

15 Examples include ethynyl, 1-propynyl, 2-propynyl, and butynyl isomers, and pentynyl isomers.

The term "heterocyclic" denotes mono- or polycarbocyclic groups wherein at least one carbon atom is replaced by a heteroatom, preferably selected from nitrogen, sulphur and

20 oxygen. Suitable heterocyclic groups include N-containing heterocyclic groups, such as, unsaturated 3 to 6 membered heteromonocyclic groups containing 1 to 4 nitrogen atoms, for example, pyrrolyl, pyrrolinyl, imidazolyl, imidazolinyl, pyrazolyl, pyridyl, pyrimidinyl, pyrazinyl, pyridazinyl, triazolyl or tetrazolyl;

saturated 3 to 6-membered heteromonocyclic groups containing 1 to 4 nitrogen atoms, such

25 as, pyrrolidinyl, imidazolidinyl, piperidyl, pyrazolidinyl or piperazinyl;

condensed saturated or unsaturated heterocyclic groups containing 1 to 5 nitrogen atoms, such as, indolyl, isoindolyl, indolinyl, isoindolinyl, indolizinyl, isoindolizinyl, benzimidazolyl, quinolyl, isoquinolyl, indazolyl, benzotriazolyl, purinyl, quinazolinyl, quinoxalinyl, phenanthradinyl, phenathrolinyl, phthalazinyl, naphthyridinyl, cinnolinyl, pteridinyl,

30 perimidinyl or tetrazolopyridazinyl;

saturated 3 to 6-membered heteromonocyclic groups containing 1 to 3 oxygen atoms, such as tetrahydrofuranyl, tetrahydropyranyl, tetrahydrodioxinyl,

unsaturated 3 to 6-membered hetermonocyclic group containing an oxygen atom, such as, pyranyl, dioxinyl or furyl;

5 condensed saturated or unsaturated heterocyclic groups containing 1 to 3 oxygen atoms, such as benzofuranyl, chromenyl or xanthenyl;

unsaturated 3 to 6-membered heteromonocyclic group containing 1 to 2 sulphur atoms, such as, thienyl or dithiolyl;

unsaturated 3 to 6-membered heteromonocyclic group containing 1 to 2 oxygen atoms and 1

10 to 3 nitrogen atoms, such as, oxazolyl, oxazolinyl, isoxazolyl, furazanyl or oxadiazolyl;

saturated 3 to 6-membered heteromonocyclic group containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms, such as, morpholinyl;

unsaturated condensed heterocyclic group containing 1 to 2 oxygen atoms and 1 to 3 nitrogen atoms, such as, benzoxazolyl or benzoxadiazolyl;

15 unsaturated 3 to 6-membered heteromonocyclic group containing 1 to 2 sulphur atoms and 1 to 3 nitrogen atoms, such as, thiazolyl, thiazolinyl or thiadiazoyl;

saturated 3 to 6-membered heteromonocyclic group containing 1 to 2 sulphur atoms and 1 to 3 nitrogen atoms, such as, thiazolidinyl, thiomorphinyl; and

unsaturated condensed heterocyclic group containing 1 to 2 sulphur atoms and 1 to 3 nitrogen

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The term "acyl" denotes carbamoyl, aliphatic acyl group or acyl group containing an aromatic ring, which is referred to as aromatic acyl, or a heterocyclic ring, which is referred to as heterocyclic acyl, preferably $C_{1-20}$ acyl. Examples of suitable acyl include carbamoyl; straight chain or branched alkanoyl such as formyl, acetyl, propanoyl, butanoyl, 2-

25 methylpropanoyl, pentanoyl, 2,2-dimethylpropanoyl, hexanoyl, heptanoyl, octanoyl, nonanoyl, decanoyl, undecanoyl, dodecanoyl, tridecanoyl, tetradecanoyl, pentadecanoyl, hexadecanoyl, heptadecanoyl, octadecanoyl, nonadecanoyl and icosanoyl; alkoxycarbonyl such as methoxycarbonyl, ethoxycarbonyl, t-butoxycarbonyl, t-pentyloxycarbonyl and heptyloxycarbonyl; cycloalkylcarbonyl such as cyclopropylcarbonyl cyclobutylcarbonyl, 

30 cyclopentylcarbonyl and cyclohexylcarbonyl; alkylsulfonyl such as methylsulfonyl and

ethylsulfonyl; alkoxysulfonyl such as methoxysulfonyl and ethoxysulfonyl; aroyl such as benzoyl, toluoyl and naphthoyl; aralkanoyl such as phenylalkanoyl (e.g. phenylacetyl, phenylpropanoyl, phenylbutanoyl, phenylisobutylyl, phenylpentanoyl and phenylhexanoyl) and naphthylalkanoyl (e.g. naphthylacetyl, naphthylpropanoyl and naphthylbutanoyl];

5  aralkenoyl such as phenylalkenoyl (e.g. phenylpropenoyl, phenylbutenoyl, phenylmethacryloyl, phenylpentenoyl and phenylhexenoyl and naphthylalkenoyl (e.g. naphthylpropenoyl, naphthylbutenoyl and naphthylpentenoyl); aralkoxycarbonyl such as phenylalkoxycarbonyl (e.g. benzyloxycarbonyl); aryloxycarbonyl such as phenoxycarbonyl and napthyloxycarbonyl; aryloxyalkanoyl such as phenoxyacetyl and phenoxypropionyl;

10  arylcarbamoyl such as phenylcarbamoyl; arylthiocarbamoyl such as phenylthiocarbamoyl; arylglyoxyloyl such as phenylglyoxyloyl and naphthylglyoxyloyl; arylsulfonyl such as phenylsulfonyl and napthylsulfonyl; heterocycliccarbonyl; heterocyclicalkanoyl such as thienylacetyl, thienylpropanoyl, thienylbutanoyl, thienylpentanoyl, thienylhexanoyl, thiazolylacetyl, thiadiazolylacetyl and tetrazolylacetyl; heterocyclicalkenoyl such as

15  heterocyclicpropenoyl, heterocyclicbutenoyl, heterocyclicpentenoyl and heterocyclichexenoyl; and heterocyclicglyoxyloyl such as thiazolylglyoxyloyl and thienylglyoxyloyl.

The term "optionally substituted" is intended to denote that a group may or may not be further substituted or fused (so as to form a condensed polycyclic group) with one or more

20  groups selected from alkyl, alkenyl, alkynyl, aryl, halo, haloalkyl, haloalkenyl, haloalkynyl, haloaryl, hydroxy, alkoxy, alkenyloxy, aryloxy, benzyloxy, haloalkoxy, haloalkenyloxy, haloaryloxy, nitro, nitroalkyl, nitroalkenyl, nitroalkynyl, nitroaryl, nitroheterocyclyl, amino, alkylamino, dialkylamino, alkenylamino, alkynylamino, arylamino, diarylamino, benzylamino, dibenzylamino, acyl, alkenylacyl, alkynylacyl, arylacyl, acylamino,

25  diacylamino, acyloxy, alkylsulphonyloxy, arylsulphenyloxy, heterocyclyl, heterocycloxy, heterocycloamino, haloheterocyclyl, alkylsulphenyl, arylsulphenyl, carboxy, carboxy ester, carboxamido, carboaryloxy mercapto, alkylthio, benzylthio, acylthio, cyano, nitro , sulfate and phosphate groups. As used herein, the term "optionally substituted" may also refer to the replacement of a -CH$_2$- group by a >C=O (carbonyl) group. Where valency constraints

30  allow, one or more optionally substituents may themselves be further optionally substituted.

Suitable optional substituents for NH include alkyl, such as methyl, ethyl, propyl and butyl; aryl, such as optionally substituted phenyl; arylalkyl, for example benzyl; heterocycle, such as pyridyl, pyrazinyl, pyrimidinyl; and acyl, such as acetyl, carbamoyl (-C(O)-O-alkyl). The

5  terms "alkoxy, "alkenoxy and "alkynoxy respectively denote alkyl, alkenyl and alkynyl groups as hereinbefore defined when linked by oxygen.

Where appropriate, any one or more of groups $R^1$-$R^4$ and/or their optional substituents may be further protected by a protecting group. Suitable protecting groups are known to those

10  skilled in the art and are described in *Protective Groups in Organic Synthesis*, T. W. Greene, John Wiley and Son (1981) and include for example alkylated and acylated oxy and amino groups and the formation of methylenedioxy groups from two vicinal or ortho-proximated hydroxy substituents.

15  The term "halogen" denotes fluorine, chlorine, bromine or iodine.

The term "aryl" denotes single, polynuclear, conjugated and fused residues of aromatic hydrocarbon ring systems.   Examples of aryl include phenyl, biphenyl, terphenyl, quaterphenyl, naphthyl, tetrahydronaphthyl, anthracenyl, dihydroanthracenyl,

20  benzanthracenyl, dibenzanthracenyl, phenanthrenyl, fluorenyl, pyrenyl, idenyl, azulenyl, chrysenyl, each of which may be further optionally substituted.

The term "haloalkyl" refers to an alkyl group, as herein before defined, substituted by one or more halogen atoms, for example, $CF_3$, $CCl_3$ or $CBr_3$.

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The term "arylalkyl" is intended to refer to an alkyl group, as herein before defined, substituted by an aryl group, as herein before defined, for example, benzyl, ethylphenyl.

In a preferred embodiment,  $R^1$ and $R^2$ together and/or $R^3$ and $R^4$ together form a group

30  of formula (a).  Suitably, when $R^1$ and $R^2$ together, or $R^3$ and $R^4$ together, independently

form a group of formula (a), U is $CH_2$ and m is 1. More preferably, the group of formula (a) is selected from one of $-(CH_2)_2-$, $-(CH_2)_3-$, $-(CH_2)_4-$, $-(CH_2)_5-$, $-(CH_2)_6-$ or $-(CH_2)_7-$. In yet another embodiment, the alkylenyl chain formed by $-(CH_2)_l-U_m-(CH_2)_n-$ is mono-or di- substituted at one or more $-CH_2-$ groups by an optional substituent, as herein

5   before defined, for example; methyl, ethyl, n-propyl, iso-propyl, hydroxy, halo, methoxy, ethoxy, iso-propoxy, acetoxy, and phenyl.

In another preferred embodiment of formula (a), U is NH, O, or S and m is 1. More preferably, $R^1$ and $R^2$, and/or $R^3$ and $R^4$, together with the nitrogen to which they are

10  attached form a group, selected from:

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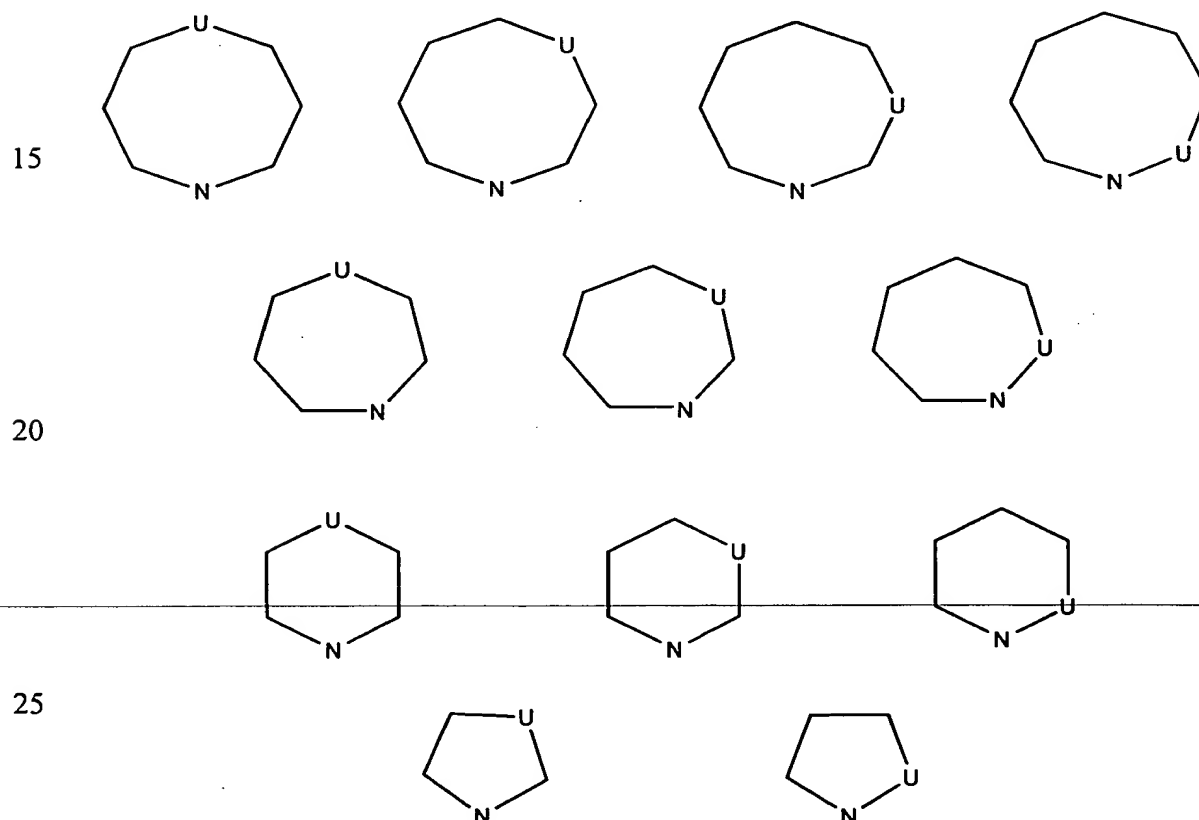
which may be optionally substituted at a carbon atom, and/or where U is NH, at the nitrogen

30  atom as hereinbefore described.

Another group of compounds which may be suitable for use in the present invention are those
of Formula (II):

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(II)
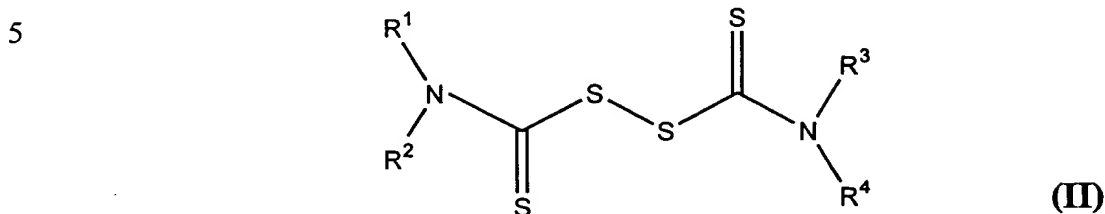
10

wherein $R^1$-$R^4$ are as defined for Formula I. In preferred embodiments of Formula (II), $R^1$
and $R^2$ and/or $R^3$ and $R^4$ together form a group of formula (a) as hereinbefore described.

The compounds for use in the present invention, suitably those of Formula (I) or (II), may
15 be administered in a single dose or a series of doses. While it is possible for the active
ingredient to be administered alone, it is preferable to present it as a composition, preferably
as a pharmaceutical composition.

Accordingly, yet another aspect of the invention contemplates a composition comprising a
20 compound capable of facilitating the disruption of a chelated metal cation domain of a protein
encoded for by an MPV gene, together with a pharmaceutically acceptable carrier, diluent or
excipient.

The carrier must be pharmaceutically "acceptable" in the sense of being compatible with the
25 other ingredients of the composition and not injurious to the subject. Compositions include
those suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or
parenteral (including subcutaneous, intramuscular, intravenous and intradermal)
administration. The compositions may conveniently be presented in unit dosage form and
may be prepared by any methods well known in the art of pharmacy. Such methods include
30 the step of bringing into association the active ingredient with the carrier which constitutes

one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

5 Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, sachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

10

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g inert diluent, preservative disintegrant (e.g. sodium starch glycolate, cross-
15 linked polyvinyl pyrrolidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying
20 proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

Compositions suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured base, usually sucrose and acacia or tragacanth gum; pastilles
25 comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia gum; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

Compositions for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter.

30

Compositions suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

5  Compositions suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bactericides and solutes which render the composition isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents.  The compositions may be presented in unit-dose or multi-dose sealed containers, for
10  example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use.  Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

15  Preferred unit dosage compositions are those containing a daily dose or unit, daily sub-dose, as herein above described, or an appropriate fraction thereof, of the active ingredient.

It should be understood that in addition to the active ingredients particularly mentioned above, the compositions of this invention may include other agents conventional in the art having
20  regard to the type of composition in question, for example, those suitable for oral administration may include such further agents as binders, sweeteners, thickeners, flavouring agents disintegrating agents, coating agents, preservatives, lubricants and/or time delay agents.  Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose, polyvinylpyrrolidone,
25  xanthan gum, bentonite, alginic acid or agar.  Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring.  Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten.  Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or
30  sodium bisulphite.  Suitable lubricants include magnesium stearate, stearic acid, sodium

oleate, sodium chloride or talc. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

In yet another aspect of the invention, there is provided a method of treating or preventing
5 a disease condition caused or exacerbated by an MPV comprising the administration of an effective amount of a compound capable of facilitating the disruption of a chelated metal cation domain of a protein encoded by an MPV gene to a mammal in need thereof.

The present invention also relates to the use of a compound capable of facilitating the
10 discreption of a chelated metal cation domain of a protein encoded b an MPV gene in the manufacture of a medicament for the treatment of prophylaxis of a disease condition caused or exacerbated by an MPV.

As used herein, the term "effective amount" relates to an amount of compound which, when
15 administered according to a desired dosing regimen, provides the desired therapeutic or prophylactic activity. The desired dosing regimen may depend on the weight, age and condition of the patient. It is within the skills and knowledge of the attending physician to determine suitable dosing regimens based thereon. Dosing may occur at intervals of minutes, hours, days, weeks, months or years or continuously over any of these periods.
20 Suitable dosages may lie within the range of about 0.1 ng per kg of body weight to 1 g per kg of body weight per dosage. The dosage is preferably in the range of 1 $\mu$g to 1 g per kg of body weight per dosage. More preferably, the dosage is in the range of 1 mg to 1 g per kg of body weight per dosage. Suitably, the dosage may be in the range of 1 mg to 500 mg per kg of body weight per dosage, for example in the range of 1 mg to 250 mg per kg of
25 body weight per dosage, or 1 mg to 100 mg per kg of body weight per dosage, such as 1mg to 50mg.

Optionally, the compounds referred to herein may also be administered in the form of their pharmaceutically acceptable salts. The term "pharmaceutically acceptable derivative" refers
30 to any pharmaceutically acceptable salt, ester, solvate, hydrate or any other compound which,

upon administration to the recipient is capable of providing (directly or indirectly) a compound as described herein. Suitable pharmaceutically acceptable salts include salts of pharmaceutically acceptable inorganic acids such as hydrochloric, sulphuric, phosphoric nitric, carbonic, boric, sulfamic, and hydrobromic acids, or salts of pharmaceutically acceptable

5  organic acids such as acetic, propionic, butyric, tartaric, maleic, hydroxymaleic, fumaric, maleic, citric, lactic, mucic, gluconic, benzoic, succinic, oxalic, phenylacetic, methanesulphonic, toluenesulphonic, benezenesulphonic, salicyclic sulphanilic, aspartic, glutamic, edetic, stearic, palmitic, oleic, lauric, pantothenic, tannic, ascorbic and valeric acids.


10 The search for anti-viral drugs is hampered when it requires assays that monitor the complete life cycle of a virus in context of the biology of the infected cell or animal. This is primarily because these *in vivo* assays are time consuming and expensive, and chemical compounds that alter the biology of the infected cells will lead to misinterpretations. In contrast, pure viral proteins expressed from cloned genes allow the development of low-cost and efficienct assays

15 specifically designed to measure the effects on the chemistry, structure and function of these proteins. These strategies have been successfully employed recently to identify drugs against several viral diseases, most notably against HIV-1. Similar efforts directed against papillomaviruses are in their infancy, even though these viruses affect several million patients a year worldwide.

20

Suitable compounds of Formula (I) and Formula (II) are those which facilitate the disruption of a chelated metal cation domain. Thus, in order to provide an initial evaluation of the efficacy of the compounds useful in the treatment of diseases or conditions caused by MPVs, the ability of these compounds to disrupt the integrity of a chelated metal cation domain,

25 thereby releasing the metal cation, offers a useful assay therefor.


This can be achieved by contacting a protein molecule, encoded by an MPV gene, containing a chelated metal cation domain with an effective amount of said compound for a time and under conditions sufficient to facilitate disruption of the chelated metal cation

30 domain and directly or indirectly determining the amount of chelated metal cation released

wherein the amount of chelated metal cation released is indicative of the disruption of the chelated metal cation domain.

Where the chelated metal is zinc, zinc release can be measured as an increase in the
5 fluorescence of the zinc-selective fluorophore TSQ (N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide) (18) in the presence of the protein and the active compound (TSQ assay).

In such an assay, the increase in fluorescence measured can be described as a percentage of the increase in fluorescence observed for a positive control compound which provides 100%
10 zinc release. A suitable positive control compound is $H_2O_2$. Preferred compounds which may be useful in the present invention are those which release at least 30% of the chelated zinc as measured by the TSQ assay. Particularly preferred compounds for use in the present invention are those which release at least 40% of the chelated zinc, more preferably at least 50%.

15

Another method of identifying compounds useful in the treatment of a disease condition caused or exacerbated by an MPV comprises contacting a protein molecule, encoded by an MPV gene, containing a chelated metal cation domain with an effective amount of said compound for a time and under conditions sufficient to facilitate disruption of the chelated
20 metal cation domain and directly or indirectly determining the absence or otherwise of binding of said protein to a ligand, wherein the absence of binding is indicative of disruption of the chelated metal cation domain.

Mutation analysis of the cysteines involved in coordinating zinc has demonstrated that zinc
25 binding is a requirement for E6 interaction with E6AP and E6BP, two coactivators of E6 mediated cellular transformation (5, 19-22). Thus, another useful assay for determining suitable compounds which may be used in the present invention measures the ability of the compound inhibit the binding of the E6 protein to E6AP and E6BP. Thus, suitable compounds which may be used in the present invention are those which are capable of
30 reducing, inhibiting or otherwise decreasing the binding interaction between the E6 protein

and E6AP or E6AB. The efficacy of compounds can be evaluated in BIACORE and GST pulldown experiments (binding assay). Preferred compounds are those which inhibit or decrease binding by at least 50%.

5 Zinc finger proteins are required for maintenance of cell viability. Preferably, the compounds for use in the present invention are specific in their ability to affect the viability of MPV containing cells, with little or no cytotoxic effects on the cellular functioning of healthy non-MPV containing cells. The viability of MPV-infected cells (and non infected cells) in the presence of compounds of Formulae (I) or (II) can be measured by incubating with the

10 tetrazolium salt WST1 (Roche Molecular Biochemicals, Manneheim Germany) and measuring the absorption readings thereof (WST1 assay).

Preferred compounds for use in the present invention produce values of at least 30% zinc release in the TSQ assay and/or inhibit binding of the E6 protein to E6AP or E6BP and/or

15 exhibit selective cytotoxicity towards MPV-infected cells.

Especially preferred compounds for use in the present invention are those which release at least 30% of chelated zinc from a protein having a chelated zinc domain as measured by the TSQ assay, and inhibit binding of the E6 protein to E6AP or E6BP as measured by the

20 herein described (BIACORE assay) and selectively inhibit cell growth of MPV-infected cells whilst having little or no cytotoxic effect on non-MPV-infected cells (for example, as determined by the WST1 assay as herein described).

Other preferred compounds inhibit binding of the E6 protein to E6AP or E6BP by at least

25 50% and are specifically toxic to MPV-infected cells.

Yet other preferred compounds release at least 50% of chelated zinc and inhibit E6 binding to E6AP or E6BP by at least 50%.

30 Suitable examples, although by no means to be considered as limiting, are illustrated below:

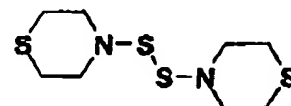**GROUP 1    TSQ > 50% AND BIACORE +  AND WST1-SPECIFICITY +**
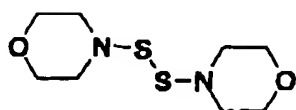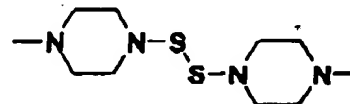
C16
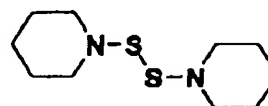
C48
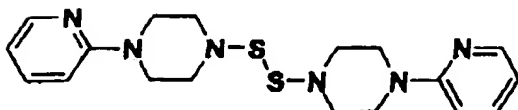
C55
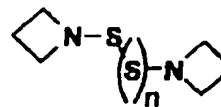
C63
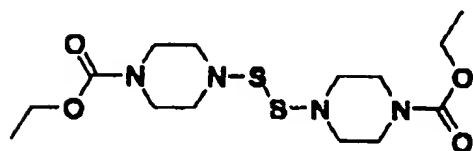
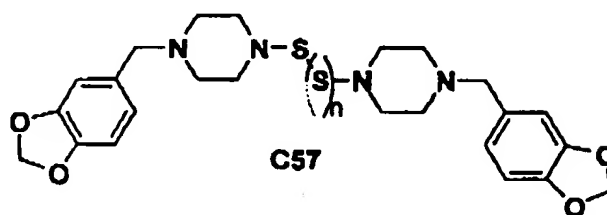**GROUP 2    TSQ > 40% AND BIACORE +  AND WST1-SPECIFICITY +**
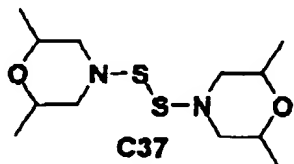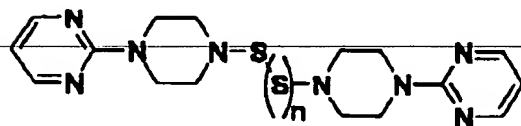
C37

C38

C39

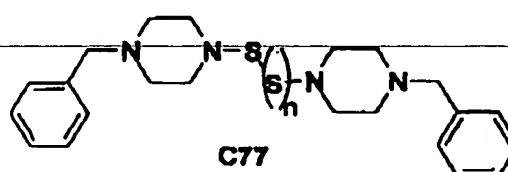**GROUP 3    TSQ > 30% AND BIACORE + AND WST1-SPECIFICITY +**
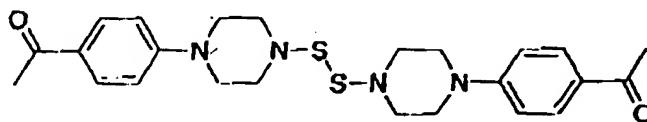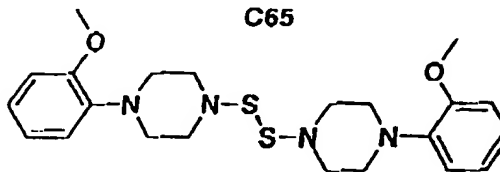
C41

C57

C75

C77

n = 1 to 5

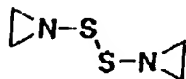**GROUP 4    TSQ < 30% AND BIACORE + AND WST1-SPECIFICTY +**
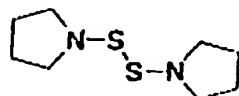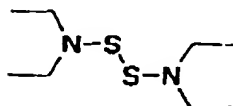


C65



C70

**GROUP 5    TSQ > 50% AND BIACORE +**
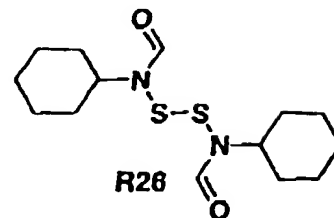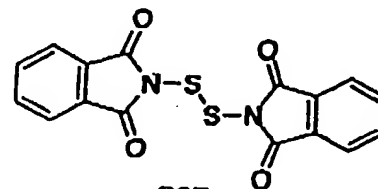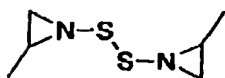


R24



C42



C49



R26
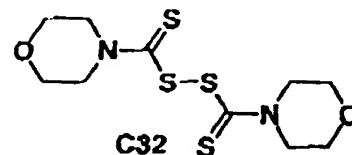


C32
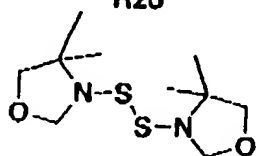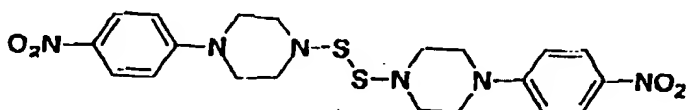


C27

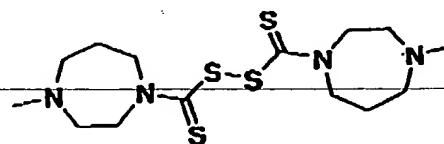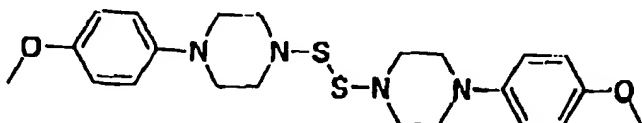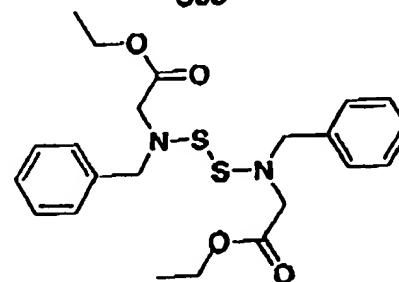**GROUP 6    TSQ > 60% OR BIACORE + OR WST1-SPECIFICITY +**



R25



C35



C82



C69



C83

The invention will now be described with reference to the following non-limiting examples

## MODES FOR CARRYING OUT THE INVENTION

5 **General**

Compounds C32 and C35 are available from Aldrich Rare Chemicals (cat # S5, 169-2) and Acros Organic (cat # 22758.60). Compound C16 is available from Tee Hai Chemicals (Singapore).

10 **Role of GSH in drug-screens**

GSH is, at concentrations of 1-10 mM in most cell types, the most abundant non-protein intracellular thiol, and it is involved in biochemical reactions that can inactivate pharmaceutical compounds. In the original TSQ assays, GSH was present at 5-10 mM.

15 Under these conditions, only a few of the compounds, including C16, were capable of releasing zinc. Increased concentrations of C16 were also required in the GST-pulldown assay, possibly to overcome the endogenous levels of GSH in the reticulolysate extracts. Similarly, in cell viability assays, C16 was only effective at concentrations of 50 $\mu$M, exceeding the amount used in TSQ assays five fold. To overcome the inactivating function

20 of GSH, higher amounts of C16 were needed *in vivo* than *in vitro*. The TSQ assay is much easier when it comes to high-throughput capabilities to identify lead compounds, while *in vivo* assays and *in vitro* assays in the presence of GSH, are useful to select compounds that reach intracellular E6 in sufficiently high concentrations and in chemically unaltered form.

25 **Expression of E6, E6AP as GST-fusion proteins**

E6, E6AP and E6BP-Glutathione S-transferase (GST) fusion proteins were prepared by using pGEX system Amersham (Pharmacia Biotech AB, Uppsala Sweden). The full length HPV-16 E6 gene was amplified via polymerase chain reaction and cloned in the vector pGeX4T2 as a Not1-Sal1 insert. A clone encoding the C-terminal 210 amino acids of

30 E6BP/ERC55 in pGEX3X was a kind gift of E.J. Androphy (5). E6AP (amino acids 213-

865), cloned in pGEX2T was a kind gift of P.M. Howley (28). These vectors were grown in the E.coli strain AB1899, induced for fusion protein expression for 4 hrs with 0.2 mM IPTG, harvested and lysed in GST-buffer (Phosphate buffered saline (PBS), 50 mM Tris pH 8.0, 0.1% Triton) with 5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride

5 (PMSF)) and 1 mg/ml lysozyme, followed by sonication. After ultracentrifugation, supernatants of bacterial lysates were incubated at 4°C on a column of gluthathione-sepharose beads (Pharmacia). Unbound, non GST-fusion proteins were eliminated by several washes with GST-buffer. For direct use of GST-fusion proteins bound on glutathione-sepharose beads in the zinc-release assay, the glutathione-sepharose beads were resuspended in PBS,

10 Tris pH 8.2. GST-fusion proteins for BIACORE analysis were eluted with elution-buffer (10 mM GSH, 50 mM Tris, PBS, pH 8.2)


## Example 1


15 **General Synthesis of Compounds of Formula (I) and (II)**

Compounds of Formula (I) can be prepared by reacting appropriately substituted amines according to the general procedure below.


Alkylation of primary amine ($R^1NH_2$, 0.1 mmol) with alkyl halide ($R^2X$, 0.1 mmol) in

20 acetonitrile (0.5 ml) and diethylamine (0.15 mmol) at 80°C for 30 min gave the secondary amine ($R^1R^2NH$) after purification on silica gel column.


A solution of the secondary amine (0.5 mmol) in petroleum-ether (10 ml) was pre-cooled to -78°C before disulfur dichloride (0.125 mmol) was added. The solution was vigorously

25 stirred for 15 minutes at -78°C and another 30 min at room temperature. Water (20 ml) was added and the desired compound was extracted into the organic phase using diethyl ether (3 x10 ml). The combined organic layers were dried over magnesium sulfate, filtered and concentrated in vacuo. The desired compound was purified on preparative TLC plate. Mixtures of amines may be used to prepare unsymmetrical compounds of Formula (I).

30

Besides the desired disulfide compound, trisulfide, tetrasulfide or pentasulfide compounds may also be obtained. A dilute reaction solution and low reaction temperature generally results in higher yield of the disulfide compound.

5



$R^1/R^2 = R^3/R^4$

$n = 1-5$
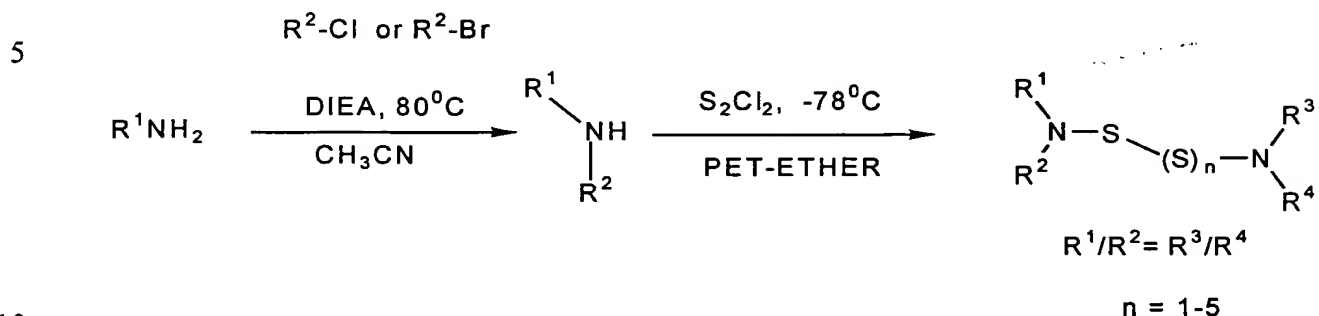
**SCHEME 1**

15

Compounds of Formula (II) may be prepared by treatment of a secondary amine (or mixtures thereof) with carbon disulfide in the presence of sodium hydroxide followed by oxidation of the resulting sodium dithiocarbamate with sodium hypochlorite.

20

**Group 1**

**C48** $(C_{10}H_{20}N_2S_2)$;

CAS: 10220-20-9

25

$^1H$ NMR (400 MHz, CDCl$_3$) δ: 1.36-1.43 (m, 4H), 1.63-1.70 (m, 8H), 2.77-2.79 (m,8H).
$^{13}C$ NMR (100 MHz, CDCl$_3$) δ: 22.8, 27.0, 57.4.
MS found 233 (M+1)$^+$

30

C55 (C$_{18}$H$_{24}$N$_6$S$_2$):

$^1$H NMR (400 MHz, CDCl$_3$) δ: 2.92-96 (m, 8H), 3.60-3.61 (m, 8H), 6.63-6.66 (m, 4H),

7.47-7.51 (m, 2H), 8.18-8.19 (m, 2H),

$^{13}$C NMR (100 MHz, CDCl$_3$) δ: 45.8, 55.4, 107.1, 113.6, 137.5, 147.9, 159.0.

5  MS found 389 (M+1).


C63 (C$_6$H$_{12}$N$_2$S$_{n+1}$):

$^1$H NMR (400 MHz, CDCl$_3$) δ: 2.09-2.28 (m, 4H), 3.79-3.83 (m, 8H)

$^{13}$C NMR (100 MHz, CDCL$_3$) δ: 17.6, 56.9, 57.5.

10  MS found for trisulfide 209(M+1)$^+$


**Group 2**


C37 (C$_{12}$H$_{24}$N$_2$O$_2$S$_2$):

15

C37 is a mixture of diastereoisomers.

$^1$H and $^{13}$C NMR were the same as reported in SYNLETT p473, August 1990.

MS found 293(M+1)$^+$


20  C38 (C$_8$H$_{16}$N$_2$S$_4$):


$^1$H NMR (400 MHz, CDCl$_3$) δ: 2.71-2.74 (m, 8H), 3.09-3.11 (m, 8H).

$^{13}$C NMR (100 MHz, CDCl$_3$) δ: 28.8, 58.2.

MS found 269 (M+1)$^+$

25


C39 (C$_{10}$H$_{22}$N$_4$S$_2$):


$^1$H NMR (400 MHz, CDCl$_3$) δ: 2.25 (s, 6H), 2.45 (broad s, 8H), 2.82-2.84 (m, 8H),

30  $^{13}$C NMR (100 MHz, CDCl$_3$) δ: 45.78, 45.82, 55.57,

MS found 263 (M+1)$^+$.

## Group 3

**C41** ($C_{14}H_{26}N_4O_4S_2$):

5  $^1$H NMR (400 MHz, CDCl$_3$) δ: 1.27 (t, J=7.1 Hz, 6H), 2.78 (broad s, 8H), 3.53 (broad s, 8H), 4.14 (q, J=7.1 Hz, 4H).

$^{13}$C NMR (100 MHz, CDCl$_3$) δ: 14.6, 44.1, 55.5, 61.6, 155.3.

MS found 379 (M+1)$^+$

10  **C57** ($C_{24}H_{30}N_4O_4S_{n+1}$):

A mixture of 2 compounds.

$^1$H NMR (400 MHz, CDCl$_3$) δ: 2.50 (broad s,8H), 3.02-3.09 (m, 8H), 3.36-3.41 (m, 4H), 5.92-5.93 (m, 4H), 6.72-6.75 (m, 4H), 6.84-6.85 (m, 2H).

15  $^{13}$C NMR (100 MHz, CDCl$_3$) δ:53.1, 56.1, 56.3, 62.4, 100.90, 107.8, 107.9, 109.3, 109.4, 122.1, 122.2, 131.7, 131.8, 146.6, 146.7 ,147.7.

MS found pentasulfide 599 (M+1)$^+$ and hexasulfide 631 (M+1)$^+$.

**C75** ($C_{16}H_{22}N_8S_{n+1}$)

20

$^1$H NMR (400 MHz, CDCl$_3$) δ: 3.08-3.10 (m, 8H), 3.90 (broad s, 8H) 6.50-6.54 (m,2H), 8.31-8.33 (m, 4H).

$^{13}$C NMR (100 MHz, CDCl$_3$) δ: 43.8, 55.1, 110.1, 157.7, 161.4.

MS found for trisulfide 423 (M+1)$^+$

25

**C77** ($C_{22}H_{30}N_4S_{n+1}$):

$^1$H NMR (400 MHz, CDCl$_3$) δ: 2.53 (broad s, 8H), 3.06-3.11 (m, 8H), 3.47 (broad s, 4H), 7.26-7.36 (10H).

30  $^{13}$C NMR (100 MHz, CDCl$_3$) δ: 53.2, 56.1, 62.7, 127.2, 128.3, 129.1, 137.8.

Multiple S observed.

## Group 4

C65 ($C_{24}H_{30}N_4O_2S_2$):

$^1$H NMR (400 MHz, CDCl$_3$) δ: 2.54 (s, 6H), 3.16 (broad s, 8H), 3.42 (broad s, 8H), 6.85-6.89 (m, 4H), 7.87-7.90 (m, 4H).
$^{13}$C NMR (100 MHz, CDCl$_3$) δ: 26.1, 47.9, 55.2, 55.6, 113.9, 130.4, 153.5.

C70 ($C_{22}H_{30}N_4O_2S_2$):
CAS: 15575-30-1
$^1$H NMR (400 MHz, CDCl$_3$) δ: 3.15 (broad s, 8H), 3.28 (broad s, 8H), 3.89 (s, 3H), 3.90 (s, 3H), 6.86-6.90 (m, 2H), 6.93-6.95 (m, 4H), 7.03-7.06 (m, 2H).
$^{13}$C NMR (100 MHz, CDCl$_3$) δ: 50.9, 55.4, 55.5, 55.9, 56.2, 111.2, 118.3, 120.9, 123.2, 140.7, 152.2.

## Group 5

C42 ($C_8H_{16}N_2S_2$):

$^1$H NMR (400 MHz, CDCl$_3$) δ: 1.83-1.89 (m, 8H), 2.88-2.91 (m, 8H)
$^{13}$C NMR (100 MHz, CDCl$_3$) δ: 24.9, 55.5.
MS found 205 (M+1)$^+$

C49 ($C_8H_{20}N_2S_2$):
CAS: 15575-30-1

$^1$H NMR (400 MHz, CDCl$_3$) δ: 1.20-1.29 (m, 12 H), 2.94-3.07 (m, 8H).

$^{13}$C NMR (100 MHz, CDCl$_3$) δ: 13.2, 51.7, 51.9, 52.0, 52.1.

MS found 208 M$^+$.

## Group 6

5

**C71** (C$_{22}$H$_{30}$N$_4$O$_2$S$_2$):

$^1$H NMR (400 MHz, CDCl$_3$) δ: 3.15 (broad s, 8H), 3.23 (broad s, 8H), 3.79 (s, 6H), 6.84-6.93 (m, 8H).

10   $^{13}$C NMR (100 MHz, CDCl$_3$) δ: 51.2, 55.5, 56.2, 114.4, 118.5, 118.7.

**C82** (C$_{10}$H$_{20}$N$_2$O$_2$S$_2$):

$^1$H NMR (400 MHz, CDCl$_3$) δ: 1.19 (s, 12H), 3.70 (s, 4H), 4.68 (s, 4H).

$^{13}$C NMR (100 MHz, CDCl$_3$) δ: 24.26, 24.28, 62.00, 79.04, 85.43

15   MS found 265 (M+1)$^+$.


## Example 2


20   **Zinc Release (TSQ Assay)**


In earlier studies, reduced glutathione (GSH) was required to elute recombinant glutathione sulfhydryl transferase E6 (recombinant (GST-E6)) protein during the purification process. However, the reducing activity of the GSH sulfhydryl groups protected GST-E6 protein from

25   the chemical attack by agents, such as the disulphide based organic compounds of Formula (I) and (II). This problem was overcome by using GST-E6 protein in the absence of free GSH but still bound to glutathione-sepharose beads. Individual assays were done in the presence of 9 μg GST-E6 protein, corresponding to a concentration of 1 μM GST-E6 protein and 2 μM bound zinc, assuming the presence of two Zn ions per protein.

30

Release of zinc from HPV-16-E6 was monitored by the change in fluorescence of the zinc-selective fluorophore TSQ (N-6-methoxy-8-quinolyl)-p-toluenesulfonamide), (Molecular Probes, Eugene, Oregon) by modification of published procedures (25, 26, 27). In a total reaction volume of 200 $\mu$l, 9 $\mu$g (1 $\mu$M) recombinant GST-E6 protein (corresponding to a

5   concentration of 1$\mu$M), bound to glutathione-sepharose beads, were incubated with 10 $\mu$M of each compound or 0.6% (170 mM) $H_2O_2$ in TSQ-assay buffer (10 mM sodium phosphate buffer pH7.0, 10% glycerol) for 2 hours at room temperature (200 $\mu$l total volume in 96-well plates).   Immediately after addition of 100 $\mu$M TSQ, the increase in fluorescence was measured on a SLT Fluostar (355 nm excitation filter and 460 nm emission filter, Tecan,

10  Salzburg).


Table 1 shows the values of TSQ fluorescence obtained.


15



20



25



30

## Table 1

| Compound | TSQ % | Compound | TSQ% |
|----------|-------|----------|------|
| DMSO | 6.1 | C57 | 31.1 |
| C16 | 63.2 | C63 | 54.8 |
| C27 | 103 | C65 | 23.7 |
| C32 | 80.9 | C69 | 0 |
| C35 | 106 | C70 | 12.4 |
| C37 | 40.3 | C71 | 0 |
| C38 | 41.8 | C75 | 37 |
| C39 | 45 | C77 | 33.8 |
| C41 | 36.8 | C82 | 28.4 |
| C42 | 50.5 | C83 | 22 |
| C48 | 54.3 | R24 | 71.0 |
| C49 | 57 | R25 | 30.9 |
| C55 | 50.6 | R26 | 54.9 |

Values given are % values of Zinc release. The relative fluorescence units (RFU) were normalized to the amount of zinc released by $H_2O_2$ (regarded as 100%). The concentration of compound used was 10 $\mu$M for C27-C35, 13 $\mu$M for C37-C83, H202 was used at 0.3% for C27-C35 and 0.2% for C37-C83.

Note:       C69 was strongly coloured (yellow / golden) at a concentration of 1 mM, all other compounds were without colour. Potentially this coloured compound interferes in TSQ-Zn fluorescence.

Values for DMSO and C16 are the average from 5 independent experiments.

## Example 3

**Binding Assays (BIACORE Assay)**

5  BIACORE allows real time analysis of bimolecular interactions without the need for isotopic or enzymatic labelling. BIACORE technology is based on the optical surface plasmon resonance (SPR), a technique that allows for detecting small changes in the refractive index on the surface of a thin gold film coated with a dextran matrix. Typically, one of the binding partners (termed the ligand) is covalently linked to the dextran matrix, while the other partner

10  (termed the analyte) is introduced in a flow passing over the surface. The change in refractive index resulting from the interaction of the molecules is expressed in resonance units (RU): a SPR response of 1000 RU corresponds to a change of the surface concentration of the analyte of 1 ng protein/mm$^2$. GST-E6AP and GST-E6BP were used as ligands and GST-E6 was used as the analyte. Controls gave the expected outcome, namely the oxidation of

15  sulfhydryl groups by $H_2O_2$ of chelating of zinc ions by EDTA completely eliminated complex formation. Further controls included using the dextran matrix alone or GST as the ligand. GST-E6 did not bind significantly to the dextran matrix or to GST (background values 100 and 200 RU), respectively.

20  Binding of GST-E6 to GST-E6BP, GST-E6AP and GST was monitored by surface plasmon resonance (SPR) on a BIACORE 2000 machine (Biacore AB, Uppsala, Sweden). Purified ligand (GST, GST-E6AP and GST-E6BP) was covalently amine coupled to a CM-5 sensor chip by activation, binding and deactivation reactions suggested by Biacore AB. Typically 6000-10000 RU of GST, E6BP and E6AP were immobilized on three difference flowcells.

25  Aliquots of purified HPV-16 GST-E6 (7 uM in 10mM GSH, 50 mM Tris/PBS buffer, pH 8.2) were incubated with either 400$\mu$M compound, or 5 mM EDTA, or 0.6% (170 mM) $H_2O_2$ for 2 hrs at room temperature. Then 10 $\mu$l of sample was injected at 1 $\mu$l/min over the three immobilized ligands using the sequential flow mode. The interactions between GST-E6 and ligands were monitored by the change of resonance signal in arbitrary units (RU). In

30  between each sample, the surfaces were regenerated with a short 1 minute pulse of 50 mM

NaOH that resulted in complete dissociation of all non-covalently bound analyte, leaving the immobilized GST-E6BP and GST-E6AP at approximately full activity. After 20 cycles of binding and regeneration, the amount of E6 binding capacity decreased approximately 18%-19% and therefore reduced the maximal amount of E6 binding. Typically complex formation

5   without compound treatment led to signals of 1540-1900RU and 1150-1400 for GST-E6 with GST-E6BP and GST-E6AP, respectively. Absence of a resonance signal, or a reduced signal was scored as an active compound.

The results for the BIACORE binding assay are shown below in Table 2.

10

15

20

25

30

## Table 2

| Compound | BIACORE (E6BP/E6AP) | Compound | BIACORE (E6BP/E6AP) |
|---|---|---|---|
| DMSO | - | C57 | + |
| C16 | + | C63 | + |
| C27 | + | C65 | + |
| C32 | + | C69 | + |
| C35 | - | C70 | + |
| C37 | + | C71 | - |
| C38 | + | C75 | + |
| C39 | + | C77 | + |
| C41 | + | C82 | + |
| C42 | + | C83 | + |
| C48 | + | R24 | + |
| C49 | + | R25 | + |
| C55 | + | R26 | + |

"+" = compound interferes in binding of E6 with E6AP and E6BP, binding is less than 50% of the corresponding E6-DMSO value.

## Example 4

### Binding of GST fusion proteins to *in vitro* translated E6 protein (GST-Pulldown Experiment)

In the BIACORE results, C16 was found to have inhibitory activity for E6 binding to both E6BP and E6AP. On this basis, it was examined whether C16 could also interfere with E6-E6BAP interaction in the GST-pulldown assay. Also, different concentrations of C16 were examined to determine the minimal concentration for inhibitory activity.

The open reading frame of HPV-16 E6, cloned into the Hind III and PstI site of the pSP64 plasmid (33), was in vitro translated with $^{35}$S-cysteine by using the TNT-SP6 Coupled Reticulocyte Lysate System as recommend by the manufacturer (Promega). All washing and binding reactions were performed with the E6BP-binding buffer described (5) but without

5    DTT (100 mM NaCl, 100 mM Tris-HCl pH8.0, 1% NP40, 0.1% nonfat dry milk and 1 mM PMSF. 40 $\mu$l of in vitro translated E6 plus 360 $\mu$l of E6BP-binding buffer were incubated for 2hrs at room temperature with test compounds at difference concentrations from 0-1 mM (dissolved in DMSO at 1%), 5 mM EDTA, and $H_2O_2$ at 0.3% (85 mM). The sample was then passed over columns captaining glutathione-sepharose beads with bound GST, GST-E6,

10   GST-E6BP or GST-E6AP proteins. The beads were heated to 95°C in 50 $\mu$l Laemmli sample buffer (BIO-RAD) with 2.5% 2-mercaptoethanol, subjected to electrophoresis on a 15% polyacrylamide gel, fixed, stained, and autoradiographed. Interference with complex formation identified reactive compounds. Desitometric quantification was performed with a BIO-RAD/GS700 imaging desitometer.

15

As shown in Fig.2, C16 inhibits E6 binding to both cellular proteins. Of the concentrations examined, concentrations from 10 $\mu$M to 100 $\mu$M provide greatest inhibitory activity.

20

**Example 5**

Determination of cell viability (WST1 assay)

25   All cell lines were obtained from the American Type Culture Collection (Manassas, VA) unless otherwise noted. SiHa (human cervical epithelial tumor line, HPV16-positive), CaSki (human cervical epithelial tumor line, HPV16-positive), HaCat (immortalized human skin epithelial cell line, HPV-negative), HeLa (human cervical epithelial tumor line, HPV18-positive), 444 (hybrid of HeLa and fibroblast, HPV18-positive) obtained from Eric Stanbridge

30   (University of California, Irvine), MCF7 (human mammary epithelial tumor cell line, HPV-

negative), HT3 (human cervical epithelial tumor cell line, HPV-negative) was obtained from the German Cancer Research Institute/ DKFZ-Heidelberg, and HepG2, (human liver epithelial tumor cell line, HPV-negative), were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100U penicillin and 1000U streptomycin.

5 Cells were allowed to attach to the surface of microwell dishes overnight and subsequently, incubated with medium containing the zinc ejecting compounds at the concentrations (10-100 uM). Viability of the cells was scored by measuring the absorption of the tetrazolium salt WST1 (Roche Molecular Biochemicals, Mannheim, Germany) in an Elisa-plate reader (Tecan, Salzburg, Austria) at a wavelength of 450 nm and a reference wavelength of 630 nm.

10

A total of 10 000 cells per well were plated on 24 well plates, after attachment overnight, they were treated 3 times in 3 days with C16 at 10 $\mu$M and 50 $\mu$M. The activity of C16 was also compared with that of C4 (azodicarbonamide) which causes ejection of zinc from HIV-1 NCp7 and is currently used in clinical trials for the treatment of AIDS. Figure 3 documents

15 that C4 did not cause growth inhibition in any of these six cell lines while C16 at 50 $\mu$M demonstrated substantial and specific inhibition of cell viability in HPV-negative cell lines as cervical epithelial tumour cells (SiHa, Caski, HeLa). C16 had little or no effect on HPV-negative cells as cervical epithelial tumour cells (HT3), mammary epithelial cancer cells (MCF7) and the immortalized skin epithelial cells (HaCat) and on the nontumorigenic (23,

20 24) HeLa-fibroblast hybrid cell line 444.

Microscopic observation (Fig. 4) demonstrates in addition the differential effect of C16 on E6-dependent cells (SiHa and HeLa) and E6-independent cells (444 and HaCat). Cell viability and the cytotoxic specificity for a number of other compounds of Formula (I) and (II) were also determined. The results are depicted in Table 3.

25

30

## Table 3

| Compound | WST1 | WST1-spec | Compound | WST1 | WST1-spec |
|----------|------|-----------|----------|------|-----------|
| DMSO | - | - | C57 | + | + |
| C16 | + | + | C63 | + | + |
| C27 | + | - | C65 | + | + |
| C32 | ++ | | C69 | - | - |
| C35 | + | - | C70 | + | + |
| C37 | + | + | C71 | + | + |
| C38 | + | + | C75 | + | + |
| C39 | + | + | C77 | + | + |
| C41 | + | + | C82 | + | |
| C42 | + | - | C83 | + | |
| C48 | + | + | R24 | + | - |
| C49 | + | - | R25 | - | - |
| C55 | + | + | R26 | + | - |

WST1:        +: cytotoxic effect in cell culture at 50 $\mu$M

WST1-spec    +: specific cytotoxic effects of compounds at 50$\mu$M in cell culture for HPV
containing cell lines as HeLa, SiHa and Caski, compared to HPV-negative cell lines
MCF7, HEPG2, (HaCat, HT3, 444).

## Example 6

### Western Blot Detection of p53 and poly-ADP ribose polymerase (PARP)

$10^6$ cells were plated on 10 cm petri-dishes with 10 ml medium and after attachment

overnight, treated with 100 $\mu$M C16 or 0.5% DMSO for one day. At the time of cell harvest most C16-treated cells were still attached to the plate. Cells were harvested using a rubber policeman and lysed in 10 mM Hepes buffer, pH 7.2, 150 mM NaCl, 0.2% Nonidet-P40 (NP40) and 1 mM PMSF, followed by centrifugation. 20$\mu$g of protein was loaded onto a

5 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and the membrane blocked with 5% nonfat dry milk in 20 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.05% Tween-20 overnight at 4 °C. The membrane was then probed with primary antibodies against p53 (Santa Cruz Biotechnology, Santa Cruz, CA), $\beta$-actin (Sigma, St. Louis, MO) or PARP (C2-10, Centre de Research du Chul, Quebec, Canada) and followed by incubation with

10 horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL). Finally, the blot was treated with an enhanced chemiluminescent detection substrate (SuperSignal, Pierce) and autoradiographed. Results are given in Figure 5.

The E6 protein forms a heteromeric complex with E6AP and P53 thereby targeting P53 for

15 degradation (30). To examine whether inhibition of E6-E6AP interaction with compounds might influence P53 levels and stability, the effect of C16 on the P53 expression was monitored (Fig.5A) and quantified with a densitometer.

Increases in P53 expression are known to be associated with programmed cell death

20 (apoptosis) (29), therefore PARP cleavage, a hallmark of apoptosis was examined in C16 treated cells. PARP cleavage was observed in C16 treated HeLa cells but not in HPV-negative HaCat cells which carry a mutant p53 gene (31) (Fig. 5B).

Those skilled in the art will appreciate that the invention described herein is susceptible to

25 variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within the spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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FIGURE 1.

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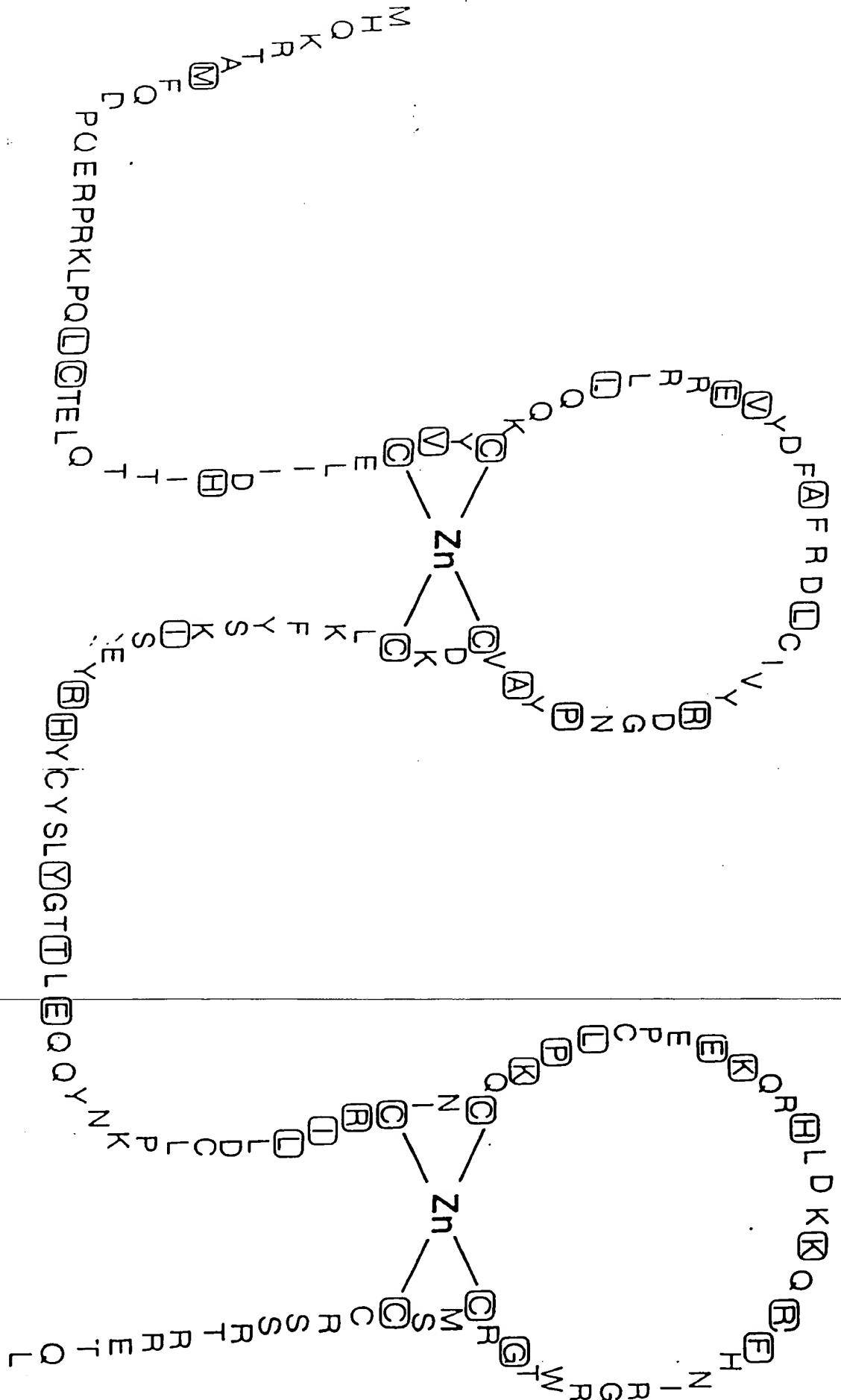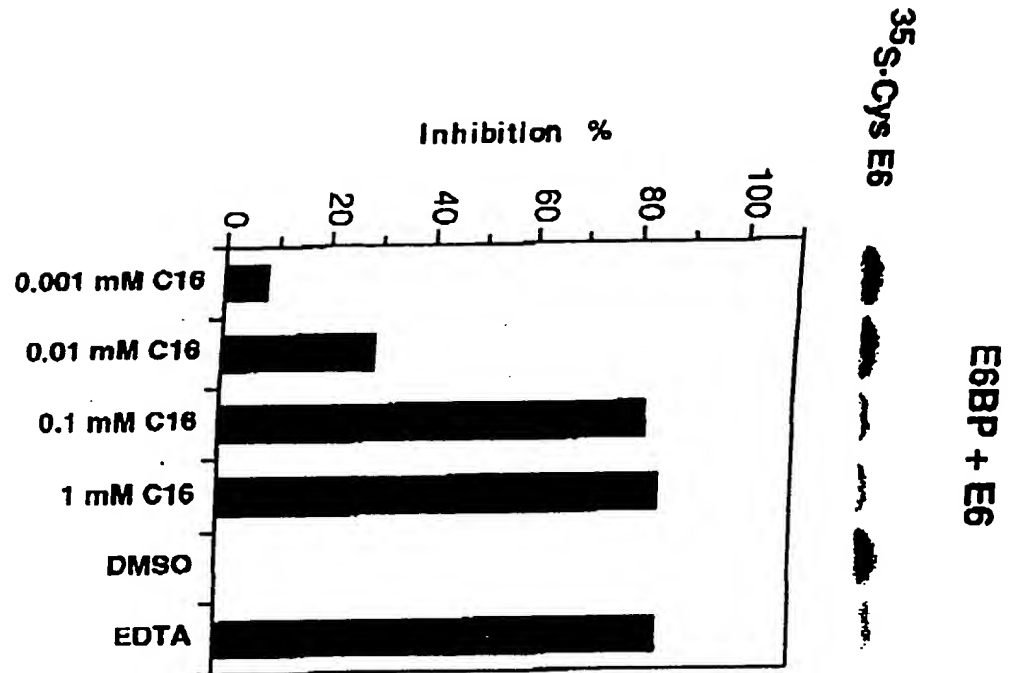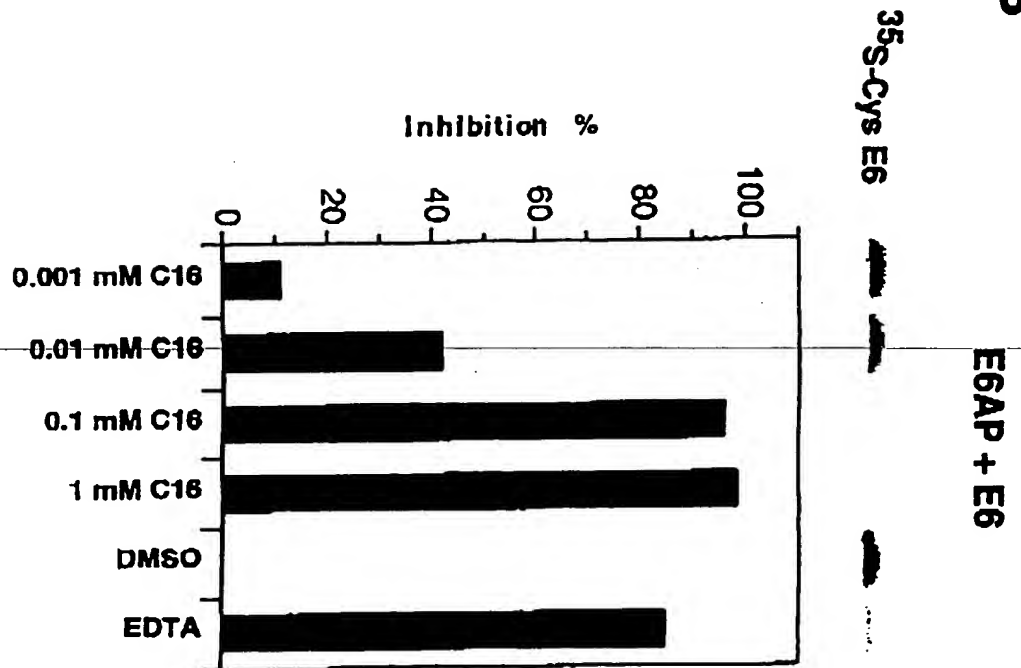


**FIGURE 2**

FIGURE 3

**FIGURE 4**

**A**

|  | HeLa | | SiHa | | MCF7 | | HaCat | |
|------|---|---|---|---|---|---|---|---|
| C16 | - | + | - | + | - | + | - | + |

p53

actin

**B**

|  | HeLa | | HaCat | |
|------|---|---|---|---|
| C16 | - | + | - | + |

PARP     116kD

89kD

**FIGURE 5**